The interaction of 1-fluoro-D-glucopyranosyl fluoride with glucosidases

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1. 1-Fluoro-D-glucopyranosyl fluoride undergoes pH-independent loss of F^- ion at a rate of $1 \times 10^{-8} s^{-1}$ at 50.0 °C, some 10^3 -fold slower than α -D-glucopyranosyl fluoride and 4×10^4 -fold slower than β -D-glucopyranosyl fluoride. 2. The (inverting) amyloglucosidase II of *Aspergillus niger* hydrolyses the difluoride according to Michaelis–Menten kinetics (K_m 34 mM and k_{cat} 0.27 s^{-1}), by apparently the same (simple) mechanism by which it hydrolyses α -D-glucopyranosyl fluoride (K_m 38 mM and k_{cat} 730 s^{-1}), rather than by the Hehre resynthesis–hydrolysis mechanism used to transform β -D-glucopyranosyl fluoride. 3. The difluoride is also a substrate for the (inverting) trehalase of pig kidney [K_m 17.3 mM and V_{max} 6.2×10^{-4} relative to α -D-glucopyranosyl fluoride (K_m 38 mM)]. 4. The quantitatively similar effect of fluorine substitution on the one-step enzymic reactions and on the non-enzymic reactions suggests that they go through similar (oxocarbenium-ion-like) transition states. 5. The difluoride is a substrate for the (retaining) β -glucosidases from *Aspergillus wentii* (A_3 enzyme) and sweet-almond meal (B isoenzyme) and for the retaining α -glucosidase from rice: comparison with the appropriate monofluoride reveals a variable rate-retarding effect of the second fluorine atom on k_{cat}/K_m that correlates with other measures of oxocarbenium ion character in the transition state. 6. The difluoride is a substrate for the (retaining) α -glucosidase from yeast, but also gives an insidious mimicry of active-site-directed irreversible inhibition, which we tentatively attribute either to formation of the non-covalent complex or to the fluoroglucosyl-enzyme increasing the well-known tendency of this enzyme to come out of solution by adsorption on the walls of the vessel.

INTRODUCTION

The interaction of fluorinated substrates with glycosidases has proved richly mechanistically informative, whether the glycosidase yields the reducing sugar product in the same anomeric configuration as the substrate, or the opposite one. Indeed, glycosyl fluorides are often the substrates of choice in determining the stereochemistry in the first place (Barnett *et al.*, 1967; Knowles *et al.*, 1988).

Although it has long been thought that inverting glycosidases operate by a single displacement by a nucleophilic water molecule (Koshland, 1953), quite the best evidence for such a mechanism comes from the discovery of Hehre's group that 'inverting' glycosidases hydrolyse glycosyl fluorides of both anomeric configurations. The pathway for the 'wrong' fluoride involves an initial transfer reaction, in which the fluorine atom of the 'wrong' fluoride mimics the nucleophilic water molecule in an approximation to the microscopic reverse of the hydrolysis reaction of

the natural substrate (Hehre *et al.*, 1979, 1982; Kitahata *et al.*, 1981; Kasumi *et al.*, 1986, 1987). Mechanisms for the hydrolysis of the natural substrate and of β -D-glucopyranosyl fluoride by glucoamylases are shown in Schemes 1 and 2.

Those glycosidases that work with retention of the anomeric configuration in the reducing sugar product operate by a double-displacement mechanism involving a glycosylated aspartic acid or glutamic acid residue as a glycosyl-enzyme intermediate, in which the anomeric configuration of the sugar is opposite to that of the substrate (Sinnott, 1987, 1990). Withers' group has shown that replacement of the C-2 hydroxy group of certain substrates of these enzymes with fluorine can, if the leaving group is good enough, result in an enzyme inactivator (Withers & Street, 1988; Withers *et al.*, 1987, 1988). The covalent glycosyl-enzyme intermediates in retaining glycosidases are reached and hydrolysed through oxocarbenium-ion-like transition states. It was thought

Scheme 1. Probable mechanism of hydrolysis of a malto-oligosaccharide by glucoamylase

Scheme 2. Probable mechanism of hydrolysis of β -glucosyl fluoride by glucoamylase

The initial product of the action of glucoamylase on β -glucosyl fluoride is thus β -maltosyl fluoride.

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that the inductive effect of the fluorine destabilized these transition states and so slowed both formation and hydrolysis; nevertheless the presence of a good leaving group such as fluoride or 2,4-dinitrophenolate in the substrate accelerated formation of the intermediate, which once formed is hydrolysed slowly, or not at all.

Recently a practical synthesis of the tetra-*O*-acetyl derivative of 1-fluoro-D-glucopyranosyl fluoride (I) was reported (Praly & Descotes, 1987). In principle either fluorine atom could be lost from compound (I) first, and thus it could be a substrate for both α - and β -glucosidases. It should therefore be possible to examine the effect of substitution of the substrate anomeric hydrogen atom by fluorine for retaining and inverting α - and β -glucosidases, by comparing the difluoride with the monofluoride. It is likely that the effect will be entirely electronic. Although there are modest steric differences between hydrogen and fluorine, in the bond lengths to carbon [C–H, 0.11 nm; C–F, 0.135 nm (Trotter, 1973)] and in van der Waals radii (H, 0.12 nm; F, 0.125 nm), it is difficult to see how such differences at the anomeric position could give rise to large kinetic effects by interaction with the enzyme protein. In the course of a glycosyl transfer the anomeric hydrogen atom has to move substantially with respect to the rest of the saccharide ring anyway. In the case of a single-displacement glycosidase, the anomeric hydrogen atom has to move through approx. 109° (from the axial to the equatorial position, or vice versa), whereas in the case of a double-displacement glycosidase it has to make two such motions, one for each step.

We therefore now report studies of the interaction of compound (I) with two representatives each of three stereochemical types of glucosidase. Inverting α -glucosidases are represented by the amyloglucosidase II of *Aspergillus niger* and pig kidney trehalase (trehalose, the natural substrate, being displayed in structure II), representative retaining β -glucosidases by the A_3 isoenzyme from *Aspergillus wentii* and the B isoenzyme from sweet almonds, and representative retaining α -glucosidases by the enzymes from rice and from yeast. [Simple inverting β -glucosidases are not easily obtained, although such enzymes are occasionally reported, e.g. by Jeffcoat & Kirkwood (1987).]

Fluorine directly substituted to an electron-deficient centre such as a glucosyl cation will destabilize it inductively, but the destabilization will be offset to some extent by conjugative electron release by the lone pairs of electrons on fluorine (Modena & Scorrano, 1973). The extent of this conjugative stabilization will depend on the extent of delocalization already present in the electron-deficient species, and is not readily predictable. We therefore also report rates of spontaneous non-enzymic loss of F^- ion from compound (I) and the α - and β -monofluorides, under comparable conditions.

MATERIALS AND METHODS

Substrates

α - and β -Glucopyranosyl fluorides were made by literature procedures (Micheel & Klemer, 1961): ^{19}F n.m.r. δ (p.p.m. with

respect to $CFCl_3$, H_2O) -146.2 and -139.1 , J_{F-H1} 53.2 and 52.0 Hz and J_{F-H2} 29.8 and 12.0 Hz respectively.

1-Fluoro-D-glucopyranosyl fluoride was prepared as follows. 1-Fluoro-2,3,4,6-tetra-*O*-acetyl-D-glucopyranosyl fluoride (0.244 g, 0.66 mmol; Praly & Descotes, 1987) was dissolved in dry methanol (20 ml), and 1.2% (w/v) sodium methoxide in methanol (5 ml) was added. The mixture was left to stand for 30 min at room temperature, and then regenerated sulphonated polystyrene ion-exchanger (Amberlite 120, H^+ form) was slowly added with shaking until the solution was neutral to litmus paper. A small amount of decolorizing charcoal was added, and the solution was filtered and evaporated to give the product, which crystallized on trituration with diethyl ether. The yield was 0.121 g (92%); m.p. $106^\circ C$ (decomp.); $[\alpha]_D^{20} +68.0^\circ$ (c 4.5 mm in water), ^{19}F n.m.r. δ (p.p.m. with respect to trifluoroacetic acid, H_2O) -3.5 and -10.5 , $J_{F(ax.)-F(eq.)}$ 148 Hz, $J_{F(ax.)-H2}$ 15 Hz (Found: C, 35.9; H, 5.36; F, 19.7; $C_6H_{10}F_2O_4$ requires C, 36.0; H, 5.34; F, 19.4%).

The three fluorinated compounds were prepared afresh by Zemplén deacetylation of the tetra-*O*-acetyl derivatives before each set of kinetic experiments. The labile β -glucopyranosyl fluoride contained maximally 8% free fluoride.

p-Nitrophenyl α - and β -D-glucopyranosides were purchased from Sigma Chemical Co. (Poole, Dorset, U.K., or St. Louis, MO, U.S.A.).

Enzymes

Purified amyloglucosidase II of *Aspergillus niger* (cf. Boel *et al.*, 1984) was a gift from Dr. Martin Schülein (Novo A/S, Bagsværd, Denmark); β -glucosidase A_3 of *Aspergillus wentii* (Legler *et al.*, 1972) was a gift from Professor G. Legler (Institut für Biochemie der Universität zu Köln, Köln, Germany). Pig kidney trehalase (lot no. 80H8500), rice α -glucosidase (lot no. 48F4018) and yeast α -glucosidase (lot no. 12F0725) were purchased from Sigma Chemical Co. Sweet-almond β -glucosidase B was isolated from the freeze-dried powder supplied by Sigma Chemical Co. (lot no. 29F4001) by medium-pressure chromatography (Waters 650 system) on a Protein Pak glass DEAE-5PW column (7.5 cm $\times$ 0.8 cm) (Nihon Millipore, Tokyo, Japan) with a linear gradient of 0–60 mM-NaCl in 20 mM-sodium acetate buffer, pH 6.0.

Buffer solutions and reaction temperatures

Non-enzymic hydrolyses were performed in 1.0 M- $NaClO_4$ buffered with the following buffers at 50 mM: pH 3, glycine; pH 4 and 5, acetate; pH 7, phosphate; pH 8, Tris (exact pH values are displayed in Fig. 1). Enzymic experiments were carried out in buffer solutions containing the buffering agent at 50 mM and Na^+ as the cation. Conditions were: for amyloglucosidase II of *A. niger*, acetate buffer, pH 5.0, at $50.0^\circ C$; for trehalase, acetate buffer, pH 5.6, at $30^\circ C$; for β -glucosidase A_3 of *A. wentii*, acetate buffer, pH 4.0, at $35.0^\circ C$; for sweet-almond β -glucosidase B, citrate buffer, pH 5.0, at $37.0^\circ C$; for yeast α -glucosidase, phosphate buffer, pH 6.8, at $25.0^\circ C$; for rice α -glucosidase, succinate buffer, pH 4.0, at $37.0^\circ C$. Enzymes were dialysed against the measurement buffer before use.

Product analysis

Products from enzymic hydrolysis of 1-fluoroglucopyranosyl fluoride, after prior removal of protein by ultrafiltration through an Amicon concentrator, were analysed on a Spectrophysics SP8800 h.p.l.c. system, fitted with an SP8430 refractive-index detector, with the use of an Alltech C_{18} column, a flow rate of 1.0 ml/min and isocratic elution with water. In addition to peaks from buffer components, peaks were detected at 3.20 min (glucosyl difluoride), 2.44 min (glucono- δ -lactone) and 1.80 min

(product also produced by incubation of the lactone in buffer in the absence of enzyme, probably gluconic acid).

The hydrolysis of the difluoride by glucoamylase II at 50 °C was monitored by ^{19}F n.m.r. with a Bruker WP 200 instrument. The solution monitored consisted of 71 mM-1-fluoro-D-glucosyl fluoride and 1.78 mg of enzyme/ml with $^2\text{H}_2\text{O}$ lock.

Kinetic methods

The kinetics of liberation of F^- ion were monitored by means of an Orion combination F^- ion electrode connected to a Corning 250 ion analyser. The electrode was calibrated with 0.1 M standard fluoride solutions (Orion, Boston, MA, U.S.A.). For all enzymic hydrolyses, and the spontaneous hydrolyses of the glucopyranosyl monofluorides, F^- ion concentration was monitored continuously, data being collected by a Dynova 286 personal computer. The reaction solution (1.0 ml) in a test tube containing a small magnetic stirrer-bar was placed in water contained in a 50 ml-capacity jacketed beaker, mounted on a magnetic stirrer, through which water maintained to within ± 0.1 °C was circulated from a Fisher 800 thermostatic bath. The temperature setting of the thermostat was adjusted by trial and error to give the required temperature in the reaction vessel. During long runs at 50 °C this was sealed with a piece of plastic film to stop evaporation. The non-enzymic hydrolysis of the difluoride at 50.0 °C was followed (to 1 % reaction) in sealed ampoules, which were removed from the thermostat at appropriate intervals, and F^- ion was assayed after the addition of TISAB III (Orion).

Spectrophotometric assays were performed on a Perkin-Elmer Lambda 6 spectrophotometer fitted with a five-position Peltier-effect thermostatically controlled cell-block. Data were captured and processed by an Equity 3 computer using the manufacturer's software (PECSS). Some spectrophotometric measurements were, however, made on a Philips PU 8800 spectrophotometer, data from which were captured and processed by a BBC Master microcomputer using the Philips kinetics software. This software was used to determine the K_i for the difluoride; initial rates were measured at four concentrations of *p*-nitrophenyl α -D-glucopyranoside substrate and six concentrations of the difluoride (including zero).

Kinetic parameters were estimated by using the non-linear least-squares software package KaleidaGraph (Synergy Software, Reading, PA, U.S.A.) run on a Macintosh SE 30 personal computer. Some first-order rate constants for non-enzymic hydrolyses were obtained graphically, by using either standard first-order or Guggenheim plots.

k_{cat} values refer to transformation of starting material, rather than liberation of fluoride ion (which would result in them being 2-fold higher for the difluoride). In the case of the very stable amyloglucosidase II of *A. niger*, it is assumed that the material supplied was pure fully active protein.

The inactivation of yeast α -glucosidase was studied by incubating the difluoride with the enzyme, and assaying 10 μl samples against *p*-nitrophenyl α -D-glucopyranoside (1.2 mM).

RESULTS AND DISCUSSION

Spontaneous hydrolyses

Fig. 1 displays first-order rate constants for hydrolysis of α - and β -glucopyranosyl fluorides and glucopyranosyl difluoride in 1.0 M- NaClO_4 at 50.0 °C as a function of pH. Although, at least in the case of the β -fluoride, there are signs of the expected acid- and base-catalysed reactions (Barnett, 1969), the hydrolysis of all three compounds at neutral pH is dominated by a pH-independent 'water' reaction corresponding to the uncatalysed loss of F^- ion [cf. a preliminary report of such a process for β -D-

Fig. 1. First-order rate constants for spontaneous hydrolyses of α -D-glucopyranosyl fluoride (■), β -D-glucopyranosyl fluoride (▲) and 1-fluoro-D-glucopyranosyl fluoride (○) as a function of pH, measured at 50 °C in 1.0 M- NaClO_4 .

The rate constants refer to loss of starting material.

galactopyranosyl fluoride (Jones & Sinnott, 1977)]. This has a rate of $4.0 \times 10^{-4} \text{ s}^{-1}$ for the β -compound and 1.0×10^{-5} for the α -compound. The difluoride is hydrolysed much more slowly, but the logarithmic response of the F^- -ion-sensitive electrode, which can measure F^- ion concentrations that are very low compared with the concentration of substrate, enables an estimate of $1 \times 10^{-8} \text{ s}^{-1}$ to be made for the rate of hydrolysis of the difluoride at 50 °C.

The 40-fold faster loss of F^- ion from β -D-glucopyranosyl fluoride than from its α -anomer represents the largest 'anti-stereoelectronic' effect to date. The 'theory of stereoelectronic control' predicts that the departure of leaving groups is favoured by the presence of antiperiplanar lone pairs of electrons on adjacent heteroatoms (Deslongchamps, 1983), but neither of the lone pairs of electrons on the ring oxygen atom is antiperiplanar to the equatorial fluorine atom of the β -compound (III), whereas one of them is antiperiplanar to the axial fluorine atom of α -glucosyl fluoride (IV), at least in the ground-state $^4\text{C}_1$ conformation of these compounds. Nonetheless F^- ion is lost from compound (III) 40-fold faster than from compound (IV). A smaller 'anti-stereoelectronic' effect has been widely observed with anionic oxygen leaving groups: loss of an anionic oxygen leaving group from the equatorial position of a pyranosyl or conformationally restricted tetrahydropyran ring is around 3–4 times faster than loss of the same leaving group from an axial position (O'Connor & Barker, 1979; Chandrasekhar *et al.*, 1983). If the theory of stereoelectronic control is abandoned (cf. Sinnott, 1988), then the results for both oxygen and fluorine leaving groups are readily rationalized in terms of the late transition state for acetal hydrolysis, which ensures that the relative reactivities of anomeric pairs of substrates are dictated largely by their relative ground-state stabilities. (The body of data taken to support the theory of stereoelectronic control can with greater economy be rationalized by the principle of least nuclear motion:

where the theory of stereoelectronic control appears to rationalize the relative reactivities of a pair of epimers, the more reactive epimer of a pair is also that in which atomic motions between ground state and transition state are the lesser. This interpretation permits exceptions, since least-motion effects are largest in reactions with central transition states and unimportant in reactions with very early or very late transition states. Acetal hydrolysis and related reactions are known to have very late transition states, and thus least-motion effects are expected to be unimportant. From the 'least-motion' viewpoint, therefore, the 40-fold greater reactivity of β - than of α -glucopyranosyl fluoride is merely a reflection of relative ground-state stabilities, whereas it is directly contrary to the predictions of the theory of stereoelectronic control.) The anomeric effect, namely the preference of electronegative substituents at the anomeric centre of pyranose rings to be axial, can be regarded either as a preferential electrostatic destabilization of the equatorial anomer or as a preferential stabilization of the axial anomer by interaction of an antiperiplanar lone pair on oxygen with the σ^* orbital of the C1-X bond (Kirby, 1983). In either case the effect is predicted to increase with the electronegativity of the substituent. The larger 'anti-stereoelectronic' effect with glucosyl fluorides than, say, glucosyl phosphates then arises simply from the greater electronegativity of fluorine compared with oxygen.

If the relative rates of hydrolysis of α - and β -glucopyranosyl fluorides merely reflect ground-state reactivities, then they cannot be taken as a guide to the relative reactivities of axial and equatorial fluorine atoms in the same molecule. Desvard & Kirby (1982) showed that the axial methoxy group of a conformationally restricted six-membered cyclic orthoester was lost faster than an otherwise equivalent equatorial methoxy group in the acid-catalysed exchange by small factors (2–11-fold) that decreased with increasing polarity of the solvent. It is reasonable to assume therefore that axial and equatorial fluorine atoms are lost at roughly equal rates in the hydrolysis of the difluoride. Therefore the non-departing fluorine atom destabilizes the transition state for the hydrolysis of β -glucosyl fluoride by $28.3 \text{ kJ} \cdot \text{mol}^{-1}$ and that for α -glucosyl fluoride by $18.5 \text{ kJ} \cdot \text{mol}^{-1}$ with respect to the parent monofluorides. This is likely to be a lower limit for true S_N1 ($D_n + A_n$) reactions, since the glucopyranosyl cation itself lives only just long enough to have a real existence in water (Bennet & Sinnott, 1986; Amyes & Jencks, 1989), so that any further destabilization will ensure it is too unstable to exist, and therefore that all reactions apparently involving it will necessarily be bimolecular. The effect of substitution of fluorine for the anomeric hydrogen atom at the reaction centre is similar to that of substitution of fluorine for the hydrogen atom at the 2-position of a 2-deoxyglucopyranosyl derivative. The data of Withers *et al.* (1989) enable a destabilization of the oxocarbenium-ion-like transition state for loss of H_2PO_4^- from α -glucopyranosyl phosphates of around $29 \text{ kJ} \cdot \text{mol}^{-1}$ to be estimated. The quantitatively similar effects of geminal and vicinal fluorine substitution, despite the fall-off of inductive effects with distance, reflect the ability of fluorine to release electrons mesomerically when it is substituted geminally to a reaction centre (cf. Chambers *et al.*, 1988).

Hydrolyses by inverting glucosidases

(i) **Amyloglucosidase II of *A. niger*.** Glucopyranosyl difluoride is a substrate for amyloglucosidase II of *A. niger*. Fig. 2 shows the variation of rate of production of F^- ion at 50.0°C with substrate concentration: the kinetics are Michaelian (K_m 34 mM and k_{cat} 0.27 s^{-1}). Fig. 3 shows that at very low substrate concentrations there is a strict proportionality between rate and substrate concentration. The hydrolysis is a process catalysed by the normal active site of the enzyme, since the K_m value is the

Fig. 2. Hydrolysis of 1-fluoro-D-glucopyranosyl fluoride by amyloglucosidase II of *A. niger* (0.89 mg/ml) in 50 mM-sodium acetate buffer, pH 5.7, at 50°C

The line is the least-squares best fit to a rectangular hyperbola.

Fig. 3. Hydrolysis of low concentrations of 1-fluoro-D-glucopyranosyl fluoride by amyloglucosidase II of *A. niger* (0.85 mg/ml) in 50 mM-sodium acetate buffer, pH 5.7

The line is the linear least-squares best fit.

same, within experimental error, as the K_i value measured against *p*-nitrophenyl α -D-glucopyranoside (36 mM). The products of hydrolysis are F^- ion, D-glucono- δ -lactone and a product of the spontaneous hydrolysis of the lactone, presumably gluconic acid.

In principle either of the fluorine atoms of glucopyranosyl difluoride could be lost first, the α -fluorine by the normal hydrolytic reaction, and the β -fluorine by the Hehre resynthesis-hydrolysis mechanism. The greater lability of β -glucosyl fluoride than α -glucosyl fluoride to spontaneous hydrolysis suggests that the normally less effective resynthesis-hydrolysis mechanism may be competitive in this particular case. We therefore monitored the reaction by ^{19}F n.m.r. The disappearance of the signals from the difluoride paralleled exactly the appearance of inorganic fluoride (at $\delta -43.5$ p.p.m. with respect to trifluoroacetic acid), with no detectable build-up of other fluorine signals. The ^{19}F signal of the 1'-fluorine atom of the anticipated transfer product, 1,1'-difluorocellobiosyl fluoride, is expected to be clearly separated from those of substrate.

Such a result is still compatible with the hydrolysis of the difluoride by the resynthesis-hydrolysis mechanism if the transfer product is hydrolysed more rapidly than it is formed, i.e. formation of the transfer product is rate-determining. In this case, however, as the substrate concentration is lowered, at some point the rate of F^- ion production must become proportional to the square of the substrate concentration. Fig. 3 shows that in

fact there is no sign of such a square dependence to the limits of our instrumentation.

The only circumstances under which these results could have been obtained if the resynthesis-hydrolysis mechanism operated would be for the first molecule of difluoride substrate to be bound with a binding constant below, say, 20 μM . If this were the case, then, since the substitution of hydrogen for a fluorine atom is a sterically conservative change, during the hydrolysis of β -glucosyl fluoride by the Hehre mechanism the enzyme should also bind the first molecule of β -glucosyl fluoride very tightly. It is known that at 24 °C *A. niger* glucoamylase hydrolyses β -glucosyl fluoride rather inefficiently (Hehre *et al.*, 1990): we attempted to measure the hydrolysis at 50 °C, but found that with 11 mM- β -glucosyl fluoride substrate an enzyme concentration of 9 mg/ml was not adequate to give a rate faster than the blank rate. It is therefore most unlikely that the difluoride is hydrolysed by the same mechanism as β -glucosyl fluoride.

The hydrolysis of α -glucopyranosyl fluoride by this enzyme obeys Michaelis-Menten kinetics with K_m 38 mM and k_{cat} 730 s^{-1} . This represents a $10^{7.9}$ -fold rate acceleration compared with the non-enzymic reaction.

The k_{cat} value for the difluoride is some $10^{3.4}$ -fold lower than for α -glucosyl fluoride (the K_m values are the same within experimental error). The hydrolysis of the difluoride is so slow that it is most unlikely that any process other than the single chemical step is rate-determining. The observation of a significant α -tritium kinetic isotope effect for hydrolysis of α -glucosyl fluoride by this enzyme [$^{3\text{H}}(V/K) = 1.19$, equivalent to an α -deuterium effect of 1.13 (Matsui *et al.*, 1989)] indicates directly that for this much better substrate the chemical step is still rate-determining.

Therefore the effect of the non-departing fluorine atom at the anomeric centre is to increase the free energy of the transition state for hydrolysis of α -glucosyl fluoride by some 21 $\text{kJ} \cdot \text{mol}^{-1}$. As argued above, this is likely to be exclusively an electronic effect. It is in fact slightly larger than the effect on the spontaneous reaction, and provides direct evidence for oxocarbenium-ion-like transition states in the mechanism of this enzyme, which is supported by the similarity of the α -deuterium kinetic isotope effects for the enzymic reaction and the spontaneous reaction [1.14 (J. Bommuswamy & M. L. Sinnott, unpublished work)]. This result can be compared with that obtained by Street *et al.* (1989), who found a defensible ($\rho = 0.90$) linear free-energy relationship between rates of acid-catalysed hydrolysis of a series of fluorinated and deoxygenated α -D-glucopyranosyl phosphates and rates of their transformations by glycogen phosphorylase, the gradient (approx. 0.8) indicating that electronic effects were somewhat attenuated in the enzymic reaction.

(ii) **Trehalase.** The behaviour of trehalase from pig kidney is similar to that of amyloglucosidase [the enzyme is now known to be an inverting one (Nakano *et al.*, 1989)]. The enzyme is comparatively unstable, and so we report only relative k_{cat} values (6.2×10^{-4} for the difluoride relative to that for α -glucosyl fluoride of 1.0). For this enzyme the K_m values are different (17.3 mM and 38 mM for the difluoride and monofluoride respectively at 30 °C), so that the destabilization of the enzymic transition state by the second fluorine atom is 18.5 $\text{kJ} \cdot \text{mol}^{-1}$ with respect to the enzyme-substrate complex but 16.6 $\text{kJ} \cdot \text{mol}^{-1}$ with respect to free enzyme and substrate. Again, a high degree of oxocarbenium ion character can be inferred.

Hydrolyses by retaining glucosidases

We expected that glucosyl difluoride would be an active-site-directed irreversible inactivator of both retaining α -glucosidases and retaining β -glucosidases, by the same mechanism as the 2-deoxy-2-fluoroglucosyl fluorides, and were surprised to discover

that it was a substrate for all four enzymes examined, although the appearance of temporary inactivation was discovered with yeast α -glucosidase. We can only speculate that the inactivation of retaining glycosidases by 2-deoxy-2-fluoroglucosyl fluorides arises as much from the removal of specific interactions of the 2-hydroxy group with the enzyme protein as from the inductive effect of the fluorine atoms.

Data for the hydrolyses, and for those of the appropriate monofluoride, are given in Table 1. For the β -glucosidase A_3 of *A. wentii* good active-site titration data with the affinity label conduritol B epoxide (Legler, 1968) allow the calculation of absolute k_{cat} values from assays with *p*-nitrophenyl β -D-glucoside. We also report absolute k_{cat} values for yeast α -glucosidase, calculated on the assumption that pure protein was 100% active (cf. Hosie & Sinnott, 1985). For the other two enzymes we report only relative k_{cat} values. The organic products from the action of the β -glucosidase A_3 of *A. wentii* on the difluoride are, as expected, gluconolactone and its hydrolysis product.

Because there are two bond-breaking steps in the action of a retaining glycosidase, and the difluoride is the only substrate known that will generate a 1-fluoroglucosyl-enzyme intermediate, it is difficult to interpret relative k_{cat} values, but from the relative k_{cat}/K_m values for monofluoride and difluoride it is possible to estimate a destabilization of the first enzymic transition state, relative to free substrate and enzyme, caused by the introduction of the second fluorine atom: this is given as $\Delta\Delta G^\ddagger$ (Table 1). It is not constant from enzyme to enzyme. The inconstancy of $\Delta\Delta G^\ddagger$ could arise in principle from real differences in the degree of oxocarbenium ion character from enzyme to enzyme, from adventitious interactions of the second fluorine atom with the enzyme protein, particularly in the binding step, or from a masking of the bond-breaking step by slower non-covalent events in the case of the monofluorides, so that measured values of $\Delta\Delta G^\ddagger$ are lower limits on the true value for the bond-breaking step. It is reasonable to assume that bond-breaking limits the rate of the much slower-reacting difluoride substrate.

However, the two enzymes in Table 1 that are characterized by highest values of $\Delta\Delta G^\ddagger$ are those for which non-covalent events are most likely to govern the value of k_{cat}/K_m for the fast-reacting monofluoride substrate, so that their already high values of $\Delta\Delta G^\ddagger$ are in fact lower limits on the true value for the bond-breaking step. For the reasons advanced in the Introduction, it is unlikely that adventitious interactions of the non-departing anomeric fluorine atom with the enzyme protein are important. Moreover, the few other measures of oxocarbenium ion character of the pyranose ring, such as α - or β -deuterium kinetic isotope effects, that have been measured follow the pattern of $\Delta\Delta G^\ddagger$ values. (α -Deuterium kinetic isotope effects arise from the substitution of deuterium for hydrogen at the reaction centre. They have their origin in the weakening of a C-H bending vibration as the hybridization of the reaction centre changes from sp^3 to sp^2 . In the case of a simple heterolytic dissociation (S_N1 or $A_n + D_n$ reaction) they thus correlate with the charge development at the transition state. If an incoming nucleophile is present, to some extent it will repel the hydrogen atom of the bending C-H bond, thereby increasing the effective force constant for the bending vibration and reducing the effect. The effect has been widely used to distinguish $A_n + D_n$ (S_N1), $A_n D_n$ (S_N2) and $A_n^* D_n$ ('borderline') reactions. Since the enzymic nucleophile in all retaining glycosidases is a carboxylate group, differing α -deuterium effects for comparable steps of different enzymes will correlate with the differing degrees of oxocarbenium ion character of the transition state for that step with different enzymes. [Different repulsion potentials for hydrogen as between chemically different nucleophiles would mask the correlation

Table 1. Kinetic parameters for the hydrolysis of 1-fluoro-D-glucopyranosyl fluoride and α - or β -D-glucopyranosyl fluoride by retaining glucosidases

Enzyme	Substrate	k_{cat}	K_m (mM)	$\Delta\Delta G^\ddagger$ (kJ·mmol ⁻¹)
Yeast α -glucosidase	αGlcF	24.8 s ⁻¹	0.93	21. ₂
	GlcF ₂	0.089 s ⁻¹	17.6	
Rice α -glucosidase	αGlcF	16 (relative)	2.3	12. ₃
	GlcF ₂	1 (relative)	17	
Sweet-almond β -glucosidase	βGlcF	2.7×10^3 (relative)	10.5	23. ₂
	GlcF ₂	1 (relative)	32	
β -Glucosidase A ₃ of <i>A. wentii</i>	βGlcF	1552 s ⁻¹	9.8	14. ₅
	GlcF ₂	6.47 s ⁻¹	11.6	

(see, e.g., Knier & Jencks, 1980).] β -Deuterium kinetic isotope effects arise from substitution of deuterium for hydrogen vicinally to the reaction centre. They have their origin in two effects. One is a small geometry-independent inductive effect, the other a larger geometry-dependent hyperconjugation. This hyperconjugative weakening of the C–H or C–³H bond is at a maximum when the dihedral angle between the bond and the electron-deficient *p* orbital on the adjacent carbon atom is 0° and zero when it is 90°.

Thus for yeast α -glucosidase the value of $\Delta\Delta G^\ddagger$ is slightly higher than that for the spontaneous hydrolysis (18.₅ kJ·mol⁻¹), yet (V/K) for both aryl glucosides and α -glucosylpyridinium ions, and hence probably α -glucosyl fluoride, is governed by a non-covalent event, possibly a conjoint change of enzyme and substrate that puts the sugar ring in the ^{2.5}B conformation (Hosie & Sinnott, 1985). The value of $\Delta\Delta G^\ddagger$, high though it is, is thus probably a lower limit. The bond-breaking step is accessible for the α -glucosylpyridinium ions, but is characterized by values of $\alpha^{\text{H}}(V)^3$ in the range 1.15–1.22 and values of $\beta^{\text{H}}(V)$ in the range of 1.08–1.13, close to the maximum of the range of such effects, and thus independently indicating a very high degree of oxocarbenium ion character for the first transition state for this enzyme. (We use the Northrop nomenclature, in which the type of isotopic substitution is specified by a preceding superscript, the kinetic parameter on which the effect is measured is in parentheses, and effects are reported conventionally, i.e. $k_{\text{light}}/k_{\text{heavy}}$.)

The value of $\Delta\Delta G^\ddagger$ for the β -glucosidase A₃ of *A. wentii* (14.₃ kJ·mol⁻¹), by contrast, is half the value for the spontaneous hydrolysis (28.₃ kJ·mol⁻¹). At the same time the action of this enzyme is characterized by values of $\alpha^{\text{H}}(V)$ around 1.10 for pyridinium salt substrates and 1.08 for aryl β -glucosides (Legler *et al.*, 1980). Both probes of enzymic transition-state structure thus give the result that the oxocarbenium ion character of the transition state of this enzyme is less.

The value of $\Delta\Delta G^\ddagger$ for the β -glucosidase B of sweet almonds is probably a lower limit, since Dale *et al.* (1986) report that k_{cat}/K_m for the hydrolysis of aryl glucosides of leaving group $\text{p}K < 8$ is governed by the diffusion-controlled binding of enzyme and substrate, and β -glucopyranosyl fluoride is as good a substrate as nitrophenyl glucosides. A highly oxocarbenium-ion-like transition state is therefore expected. In accord with this, the value of $\alpha^{\text{H}}(V)$ for the hydrolysis of 3,4-dinitrophenyl glucoside is 1.25 ± 0.01 (M. L. Sinnott & I. J. L. Souchard, unpublished work). (This effect could apply to the hydrolysis of the glycosyl-enzyme or to its formation, but, since the enzyme has transglucosidase activity, if one transition state has a high degree of oxocarbenium ion character, by microscopic reversibility they both must have.)

It therefore appears that $\Delta\Delta G^\ddagger$ values might be a useful and

Fig. 4. Apparent active-site-directed inactivation of yeast α -glucosidase by 1-fluoro-D-glucopyranosyl fluoride (6.19 mM) in 50 mM-sodium phosphate buffer, pH 6.8, at 25 °C

Logarithms of percentage residual activity are plotted against time: the lines are the least-squares best fits. The Δ symbols refer to the inactivation in the absence of a protecting agent, the $\square$ symbols to inactivation in the presence of 38.5 μM -1-deoxynojirimycin (K_i 23 μM ; Hosie & Sinnott, 1985). If the inactivation is assumed to be active-site-directed, these data give an apparent binding constant for the difluoride of 14 mM, compared with the K_i for hydrolysis of 17.6 mM.

readily determined measure of oxocarbenium ion character in the transition state of a glucosidase.

Apparent inactivation of yeast α -glucosidase

Yeast α -glucosidase was inactivated by glucosyl difluoride in a manner that at first suggested that indeed it was an inactivator similar to the 2-deoxy-2-fluoroglucosyl fluorides. Fig. 4 shows the time courses of loss of α -glucosidase activity when the enzyme is incubated with an excess of the difluoride; the inactivation is apparently first-order, and protection by a reversible inhibitor of catalysis (deoxynojirimycin) is observed. Controls were stable over the time of all these experiments. For any one set of experiments the derived first-order rate constants showed the expected saturation-dependence on difluoride concentration, but between one set of experiments and another the derived parameters varied by up to 3-fold. This variation was not trivially due to different purities of different batches of difluoride, since constants for hydrolysis by both this enzyme and others could be reproduced. Complete inactivation was never seen. Dialysis of 'inactivated' enzyme resulted in a non-first-order recovery of activity, again in an irreproducible way. Yeast α -glucosidase is a notoriously 'sticky' enzyme that is sold admixed with BSA stabilizer. We speculate that the ability of the enzyme to stick to the walls of containers is increased in complexes with

the difluoride or in the fluoroglucosyl-enzyme, and report our data merely to emphasize the hazards of claiming active-site-directed irreversible inhibition on the basis of kinetic measurements alone.

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